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Genetic Diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia

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Abstract: The genus *Ostreopsis* is an important component of benthic and epiphytic dinoflagellate assemblages in coral reefs and seaweed beds of Malaysia. Members of the species may produce toxins that contribute to ciguatera fish poisoning. In this study, two species have been isolated and cultured, *Ostreopsis ovata* and *Ostreopsis lenticularis.* Analyses of the 5.8S subunit and internal transcribed spacer regions ITS1 and ITS2 of the ribosomal RNA gene sequences of these two species showed that they are separate species, consistent with morphological designations. The nucleotide sequences of the 5.8S subunit and ITS1 and ITS2 regions of the rRNA gene were also used to evaluate the interpopulation and intrapopulation genetic diversity of *O. ovata* found in Malaysian waters. Results showed a low level of sequence divergence within populations. At the interpopulation level, the rRNA gene sequence distinguished two groups of genetically distinct strains, representative of a Malacca Straits group (isolates from Port Dickson) and a South China Sea group (isolates from Pulau Redang and Kota Kinabalu). Part of the sequences in the ITS regions may be useful in the design of oligonucleotide probes specific for each group. Results from this study show that the ITS regions can be used as genetic markers for taxonomic, biogeographic, and fine-scale population studies of this species.

Key words: *Ostreopsis ovata,* benthic dinoflagellates, genetic diversity, rRNA, HAB species, Malaysia.

INTRODUCTION

Benthic and epiphytic dinoflagellates are important components of microalgal assemblages in tropical and subtropical waters. Certain species in this group of dinoflagellates are known to produce several potent toxins that may be involved in ciguatera fish poisoning. The occurrence of this group of dinoflagellates in Malaysian waters has never been formally documented. Samplings that we have carried out, however, show that they are common in seaweed beds and coral reefs in various parts of Malaysia (unpublished data). These include species of *Gambierdiscus, Ostreopsis, Coolia, Prorocentrum,* and *Amphidinium.* Through these sampling efforts we know that at least two species of *Ostreopsis* exist in Malaysian waters, *Ostreopsis lenticularis* and *Ostreopsis ovata.*

The benthic/epiphytic dinoflagellates have limited capabilities for dispersal. They are rarely encountered in the plankton. Dispersal might occur via rafting on pieces of seaweed that become detached from the substratum. The limited dispersal capability means that the probability of genetic exchange between widely separated populations of

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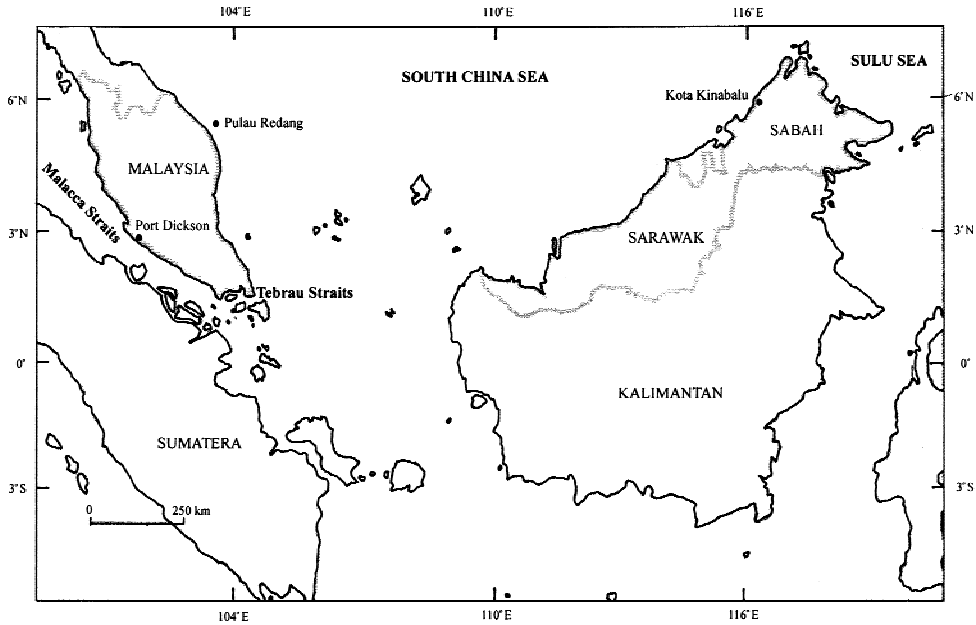

Figure 1. Sites from which the *Ostreopsis* clones used in this study were originally isolated.

these dinoflagellates is low. Even when cells from different populations do mix, it is not known if they could sexually reproduce. In the case of *O. ovata,* for example, it is still uncertain if sexual reproduction occurs. It may be postulated that geographically separated populations of *O. ovata* in Malaysia's marine waters evolve independently of each other over time. Random mutations may occur within a population and would be propagated within the population through asexual reproduction. In this study we used the ribosomal RNA gene sequences to analyze the interpopulation and intrapopulation genetic diversity of this species in Malaysian waters.

Materials and Methods

Isolation and Establishment of Clonal Cultures

Seaweed samples were collected from various parts of Malaysia for the isolation of epiphytic dinoflagellates. *Ostreopsis ovata* was found in samples from Port Dickson, Pulau Redang, and Kota Kinabalu (Figure 1). The red and brown seaweeds *Sargassum* spp., *Padina* spp., *Turbinaria* spp., and *Dictyota* spp. were hand-collected into plastic bags by snorkeling and scuba diving. In the laboratory, the samples were vigorously shaken and then sieved through 120-μm and 20-μm Nitex sieves. Materials retained by the 20-μm sieve were resuspended in filtered seawater and examined under a stereoscope for cell isolation by micropipetting. Clonal cultures of the species were established in ES-DK medium (Kokinos and Anderson, 1995). Cultures were maintained at 26°C under a cycle of 14 hours of light and 10 hours of dark. The cultures were not axenic.

Morphological Observations

Species identification was based on morphological descriptions given by Fukuyo (1981) and Faust et al. (1996). For normal light microscopy the cultures were fixed in 4% glutaraldehyde. Cell dimensions were determined by measuring the dorsoventral diameter and transdiameter of 25 fixed cells using an eyepiece micrometer at magnification ×400.

Thecal morphology and plate tabulation was examined under an epifluorescence microscope with calcofluor-white staining. Cells were harvested from exponentially growing cultures by centrifugation (2000 *g* for 5 minutes) and lysed by adding sterile distilled water (ddH_2O) and a few drops of 1% calcofluor-white solution were added. The cells were stained for 10 minutes and observed under a Nikon epifluorescence microscope using a ×40 fluorescence objective.

Nucleic Acid Extraction

Cells were harvested from approximately 125 to 150 ml of midexponential batch culture by centrifugation (3000 *g* for 5 minutes). The cell pellet was rinsed once with ddH_2O. The

cells were resuspended in NET buffer containing 1% sodium dodecyl sulfate (SDS), 15 mM NaCl, 10 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.0, and 10 mM Tris-HCl (pH 7.5) and incubated at 65°C for 30 minutes. Cetyltrimethylammonium bromide (CTAB) was added to a final concentration of 1%, and the sample was adjusted to contain 0.8 M NaCl. The mixture was incubated at 65°C for 10 minutes and extracted once with chloroform–isoamyl alcohol (24:1). The sample was then extracted using standard phenol-chloroform procedures. DNA was precipitated by the addition of 2 volumes of ethanol (EtOH) and 0.1 volume of 3 M sodium acetate (NaOAc, pH 5.0). The DNA pellet was then rinsed with 80% EtOH, and dissolved in 50 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0).

Polymerase Chain Reaction Amplification and Sequencing

The polymerase chain reaction (PCR) primers used in this study were obtained from Operon Technologies, Inc. (Alameda, Calif.). Primers ITS1F (5′-TCGTAACAAGGTTTCCGTAGGTG-3′) and ITS1R (5′-ATATGCTTAAGTTCAGCGGG-3′) were partly based on ITSA and ITSB of Adachi et al. (1994). Primers ITSc (5′-CACCGCCCGTCGCTCCTACCG-3′) and ITSd (5′-GCATATCCTTGTTCATTCGC-3′) were designed based on published nucleotide sequences of 18S and 28S subunits of dinoflagellate rRNA genes (Lenaers et al., 1989; Scholin, 1994).

PCR was typically carried out in a 50-µl reaction mixture containing 2 U *Taq* polymerase (Promega, Madison, Wis.), 200 µM dNTPs (Promega), 1 × PCR buffer, 2 mM $MgCl_2$, 0.75–1 µM each primer, and 50–100 ng total genomic DNA. PCR amplifications were performed as follows: 2 minutes initial denaturing at 95°C, annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 50°C for 90 seconds, and extension at 72°C for 3 minutes. The PCR was carried out on a PTC-150 MiniCycler (MJ Research Inc., Waltham, Mass.). The PCR products were further purified using QIAquick purification columns (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. The purified products were stored at −20°C.

The purified PCR products were directly sequenced using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystem, Foster City, Calif.). Sequencing was carried out on an ABI 377 automated sequencer (PE Applied Biosystem). Sequencing for each sample was carried out on both strands.

Phylogenetic Reconstruction and Data Analysis

The sequences obtained were initially evaluated by running them through the BLAST program (Altschul et al., 1990). Multiple alignments were constructed using the CLUSTAL X program (Thompson et al., 1997) and subsequently aligned by eye. The termini of rRNA coding regions and internal transcribed spaces (ITS) regions were determined by comparison with published sequences (McNally et al., 1994; Adachi et al., 1996) and confirmed by construction of the rRNA secondary structures. Data were analyzed by neighbor joining (NEIGHBOR; NJ) and maximum parsimony methods (DNAPARS; MP) in PHYLIP version 3.5c (Felsenstein, 1993). Sequence divergences were calculated using the Kimura (1980) 2-parameter model in DNADIST of PHYLIP. The confidence limits of tree topology were tested by bootstrapping with 1000 replications (Felsenstein, 1985). The most parsimonious tree was determined by randomizing the input order 10 times. The distance tree and parsimony tree inferred from ITS1-5.8S-ITS2 sequences were rooted with *Ostreopsis lenticularis* (clone OIPR01) as the outgroup.

The sequences were also analyzed using DnaSP (DNA sequence polymorphism version 3.0; Razos and Razos, 1999). The genotype diversities were calculated based on Nei (1987). Gene flow was estimated from the F_{ST} value (Hudson et al., 1992) as determined by the Gene Flow algorithm in DnaSP.

Results

Morphological Observations

Twenty-five clones of *Ostreopsis* spp. from Malaysian waters have been isolated and cultured to date (Table 1). All but one of the isolates resembled *O. ovata* in cell size as described by Fukuyo (1981) and Faust et al. (1996), although there were slight morphological differences. Only one clone could be designated as *O. lenticularis* on the basis of cell size and thecal pore.

Observation of thecal plate morphology by calcofluor staining confirmed these species as *Ostreopsis* (Figure 2). The Kofoidian plate tabulation is similar to that for other *Ostreopsis* spp. already described (Faust et al., 1996): i.e., P_o, 3′, 7″, 5‴, 1p, and 2⁗. However, fine-scaled structures of the theca such as the ventral plate, ridge plate, sulcus plates, and cingulum plates were not clearly visible in the calcofluor-stained cells.

Table 1. Species and Clone Numbers of *Ostreopsis* Species Used in This Study, with Sampling Locations and GenBank Accession Numbers for the rRNA Gene Sequences for Each Clone

Species and clone no.	Location	Month collected (1997)	GenBank accession no. ITS1-5.85-ITS2 regions	GenBank accession no. rDNA
Ostreopsis ovata				
OvPD04	Port Dickson	May	AF076217	
OvPD06	Port Dickson	May	AF218455	
OvPD07	Port Dickson	May	AF218456	
OvPR01	Pulau Redang	July	AF218457	
OvPR02	Pulau Redang	July	AF218459	
OvPR03	Pulau Redang	July	AF076218	
OvPR04	Pulau Redang	July	AF218458	AF244939*
OvSA02	Kota Kinabalu	October	AF218460	AF244940†
OvSA04	Kota Kinabalu	October	AF218461	
OvSA06	Kota Kinabalu	October	AF218463	
OvSA09	Kota Kinabalu	October	AF218462	
OvSA10	Kota Kinabalu	October	AF218464	
Ostreopsis lenticularis				
O1PR01	Pulau Redang	July	AF218465	AF244941†

*18S subunit.
†28S subunit.

The Kota Kinabalu clones were of similar size and shape to the Pulau Redang clones. The isolates from Port Dickson were smaller and much rounder (Table 2). Even though the cultures are clonal, two size classes of cells tend to develop after a prolonged period. In the case of the Kota Kinabalu isolates, the large cells ranged from 45 to 55 μm in dorsoventral diameter and 31 to 39 mm in transdiameter, whereas the small cells ranged from 32 to 40 μm in dorsoventral diameter and 22 to 30 μm in transdiameter. It is not known if the smaller cells are gametes. Morphologically the two size classes are similar.

Ribosomal RNA Gene Sequence Analysis

Complete sequences of the 5.8S subunit of the rRNA gene and ITS regions from 12 clones of *O. ovata* were amplified and sequenced. The 5′ and 3′ termini of 5.8S rRNA and the beginning and ending of ITS regions were determined by sequence comparisons and examination of the secondary structure. The most probable secondary structure of 5.8S rRNA and partial 28S rRNA (domain 1) (Figure 3) were constructed on the basis of the model proposed for *Prorocentrum micans* (Van der Auwera and De Wachter, 1998).

Alignment of the sequences revealed similar lengths of these regions among the isolates (Figure 4). The primers, the 3′ end of 18S rRNA gene, and the 5′ end of 28S rRNA gene are not included in this alignment analysis. The 5.8S region was 159 bp long in all isolates of *O. ovata,* and the sequences were almost identical, differing at only 4 nucleotide positions. The length of the 5.8S region is almost similar to that of other dinoflagellates that have been studied.

The sizes of the ITS regions were quite varied. The lengths of both ITS1 and ITS2 were different among isolates from different populations. The ITS1 spacer was 90 bp long in both Pulau Redang and Kota Kinabalu isolates but only 87 bp long in Port Dickson isolates. The ITS2 of Port Dickson isolates was 4 to 5 bp shorter than that of Pulau Redang and Kota Kinabalu isolates (Table 3). The ITS1 and ITS2 lengths are significantly shorter than those of other dinoflagellates that have been reported (Adachi et al., 1996).

The ITS1-5.8S-ITS2 sequence of *O. lenticularis* (clone

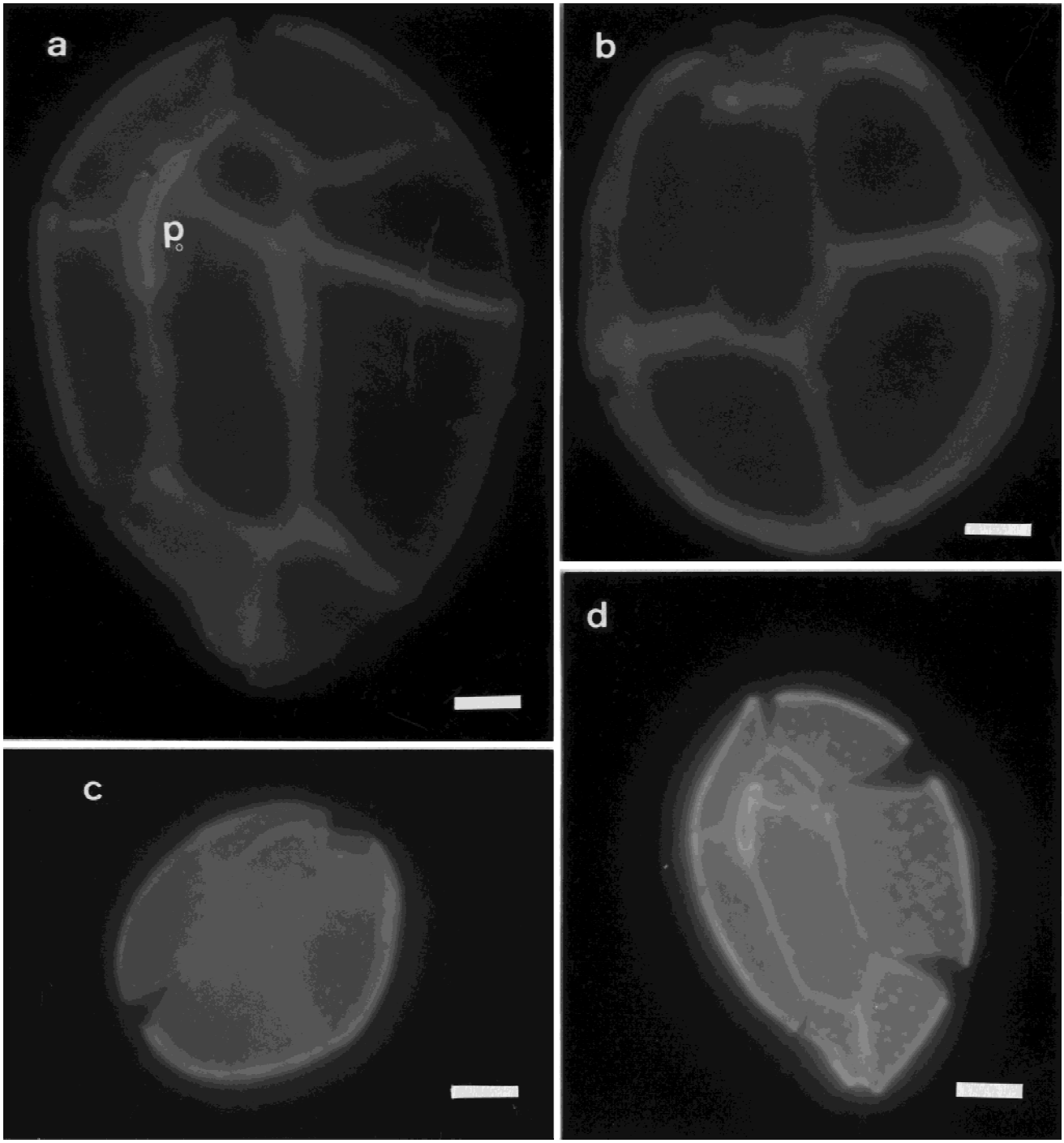

Figure 2. Calcofluor-stained cells viewed under epifluorescence microscopy. P_o indicates the apical pore. Scale bar = 10 μm: (**a**) *Ostreopsis lenticularis* epitheca; (**b**) *O. lenticularis* hypotheca; (**c**) *Ostreopsis ovata* hypotheca; and (**d**) *O. ovata* epitheca.

OIPR01) was also included in the analysis. However, the sequence aligned poorly with *O. ovata* sequences. Sequence comparison of the two species showed very high divergence of 78.5% to 82.8%. Thus the 5.8S rRNA secondary structure was constructed to determine the 3′ and 5′ ends of the regions, and the sequences were aligned manually to match up the unambiguously aligned positions. Results from the alignment clearly showed high levels of nucleotide differences between the two species, especially in the ITS1 and ITS2 regions (data not shown). However, the alignment

Table 2. Comparison of Morphological Characters of *Ostreopsis ovata* and *Ostreopsis lenticularis* as Observed by Light Microscopy

		O. ovata		
Character	*O. lenticularis*	Port Dickson	Kota Kinabalu	Pulau Redang
Cell shape	Lentil-like	Oval to tear-like	Tear-like	Tear-like
Dorsoventral diameter	64–76 μm	33–41 μm	32–55 μm	44–48 μm
Transdiameter	52–65 μm	24–34 μm	22–39 μm	33–37 μm
Apical pore	Curved	Curved	Straight	Straight
Trichocyst pores	Round; equal in size	Round	Round; unequal in size	Round

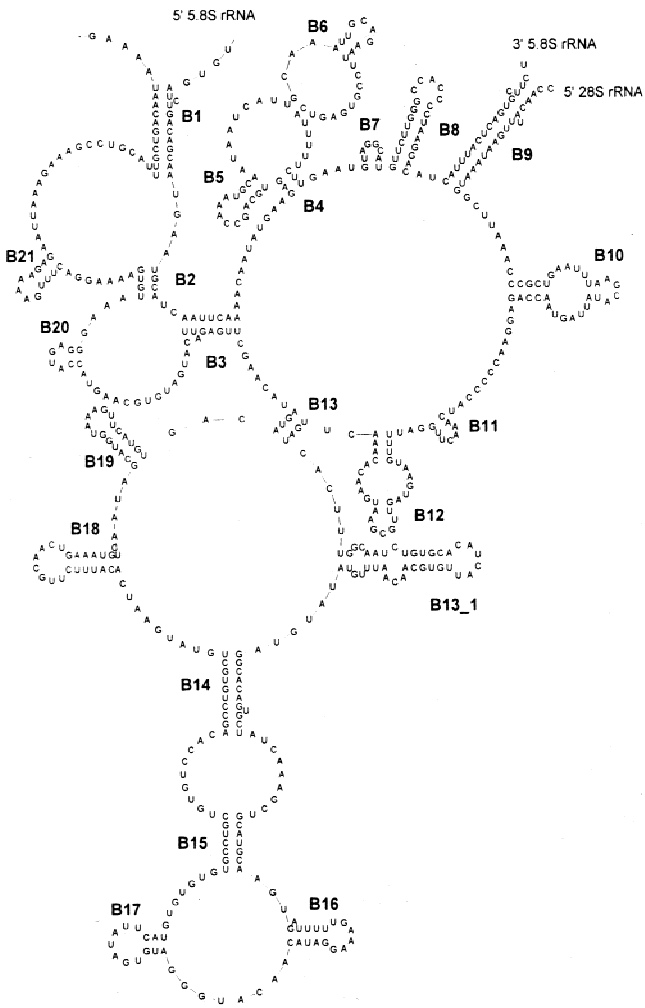

Figure 3. Proposed secondary structure of the 5.8S and 28S subunits of the rRNA gene of *Ostreopsis ovata.* The structure was used to determine the termini of the 5.8S coding region.

revealed that the length of 5.8S rRNA was conserved with only one extra base pair in the *O. lenticularis* sequence (Table 3). The results also showed that in both species ITS2 is larger than ITS1.

Genetic Diversity of *Ostreopsis ovata*

Sequence analysis of 12 clones of *O. ovata* revealed the existence of genetic variation within this species. The distance tree (NJ) inferred from ITS1-5.8S-ITS2 rDNA sequences showed two distinct groups of isolates, a Malacca Straits group (Port Dickson isolates) and a South China Sea group (Kota Kinabalu-Redang isolates) (Figure 5). Maximum parsimony analysis (MP) also revealed a topology identical to the distance tree (data not shown). Port Dickson clones formed a group that was clearly separated from the other isolates with bootstrap support of 99.6% (in NJ) and 100% (in MP) for this clade. The Kota Kinabalu and Pulau Redang isolates together formed a clade that was strongly supported (97.0%). The grouping of both populations together was also well supported in MP (94.3%).

The divergence between Port Dickson isolates and Kota Kinabalu–Pulau Redang isolates ranged from 7% to 9% (Table 4). However, the level of sequence divergence between clones from the same locality was very low (0%–0.3%).

The intraspecific heterogeneity among populations of Port Dickson, Kota Kinabalu, and Pulau Redang are very low with nucleotide diversity index (P_i) of 0.002, 0.009, and 0.003, respectively. However, the interpopulation diversity between the Port Dickson population and other populations indicated approximately 50% of genetic differentiation (Table 5). Only 1% of the nucleotide diversity was attributable to the differences between Pulau Redang and Kota Kinabalu populations. The relatively high F_{ST} values across the three sampled areas suggest that the rate of gene flow among these populations is very low (Table 5).

Discussion

Currently 6 species of *Ostreopsis* have been described (Fukuyo, 1981; Faust et al., 1996). In the present study, all

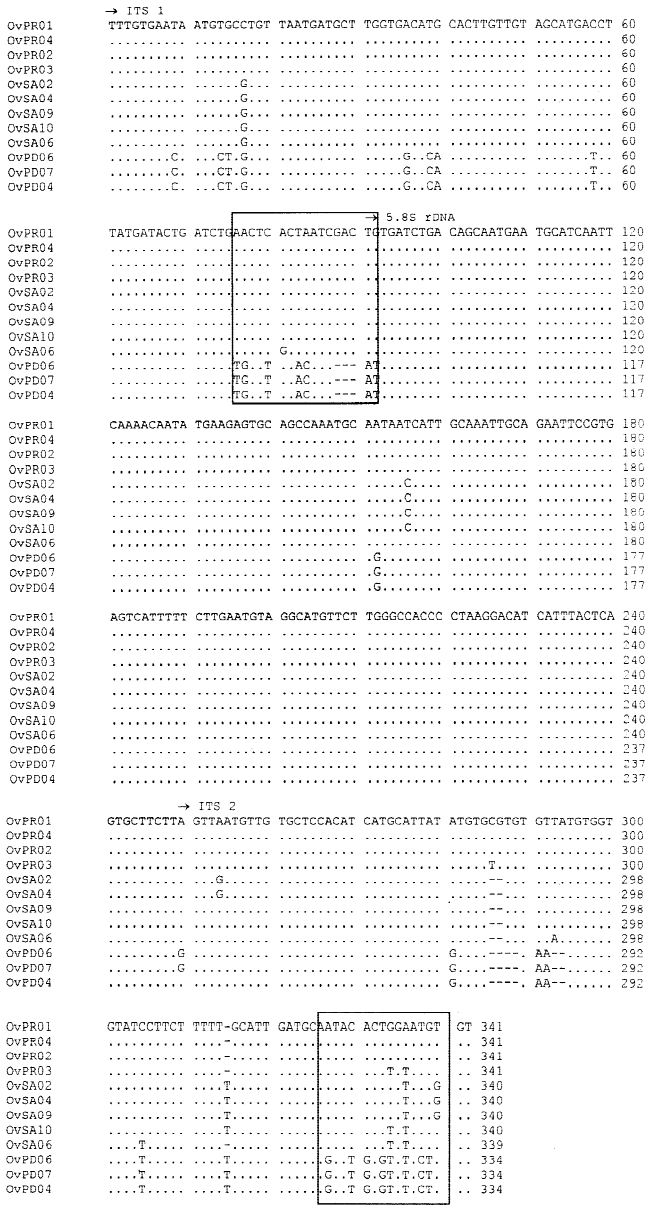

Figure 4. Multiple alignment of the 5.8S rRNA gene and ITS sequences from 12 clones of *Ostreopsis ovata* from Pulau Redang (OvPR), Kota Kinabalu (OvSA), and Port Dickson (OvPD). Dot (·) indicates identical base, while dash (–) indicates deletion and alignment gap. Boxed regions show signature sequences specific to two distinct groups of *O. ovata* from Malaysia.

but one of the isolates resembled *O. ovata* in morphology. Minor morphological differences observed within the isolates were insufficient to differentiate these isolates that originated from different sampled areas. Results from this study also showed that plasticity in morphology of this species poses a significant problem in morphospecies determination.

Table 3. Size of the 5.8S rRNA Gene and ITS Regions (in base pairs) of *Ostreopsis ovata* and *Ostreopsis lenticularis* from Different Locations

Species	ITS1	5.8S	ITS2	Total length
Ostreopsis ovata				
Pulau Redang clones	90	159	92	341
Port Dickson clones	87	159	87–88	333–334
Kota Kinabalu clones	90	159	90–91	339–340
Ostreopsis lenticularis	76	160	141	377

Molecular analysis based on ITS1-5.8S-ITS2 rDNA sequences clearly indicates that the *O. ovata* populations from various parts of Malaysia were genetically structured. Data analysis of genetic heterogeneity revealed a degree of divergence among them. In addition, there was a consistency in genetic relationship with respect to geographic areas. The Port Dickson population appeared genetically distinct from Kota Kinabalu and Pulau Redang populations, but the Kota Kinabalu and Pulau Redang populations seemed to share the same degree of genetic divergence between populations. On the basis of results from the phylogenetic analysis, it may be proposed that *O. ovata* from Malaysian water consists of two distinct groups, the Malacca Straits group (SM) and the South China Sea group (LCS). The estimates of F_{ST} indicate the absence of gene flow between the two groups, and it is likely that genetic exchange between the Port Dickson population and the other two populations does not occur. The Malacca Straits and South China Sea are separated by the Malaysia Peninsula and connected only by the narrow Tebrau Straits. It is thus likely that mixing between the two bodies of water is very limited. In addition, the habits of *O. ovata* may further enhance the separation effect. In this respect a similar type of study on planktonic species would be interesting.

In population genetics theory, genetic drift occurs with the assumption that the population undergoes sexual reproduction. The ability of *O. ovata* to reproduce sexually is still unclear. Resting cysts of the closely related species *Coolia monotis* have been reported in cultures (Faust, 1992), but cysts of *O. ovata* have not yet been found.

Kota Kinabalu and Pulau Redang isolates seem to form two different groups, as illustrated in both phylogram and cladogram, but the data obtained are not sufficient to separate them into two distinct groups. The small degree of

Figure 5. a: Distance tree (neighbor-joining) inferred from the 5.8S rDNA and ITS sequences of *Ostreopsis ovata* and *O. lenticularis* using the closely related species *Coolia malayense* as the outgroup. **b:** Magnified view of the *O. ovata* branches to show the percentage of bootstrap support for each branch from 1000 replicates. Values <50% are not shown.

genetic variation observed between Kota Kinabalu and Pulau Redang isolates also revealed no significant difference between these populations (1.19%–1.80% divergent). It cannot be discounted, of course, that divergence values between these two populations could be higher if more clones were analyzed. The estimated F_{ST} value suggests some degree of genetic exchange between these populations; hence, migration may occur between them.

The dispersal capability in benthic/epiphytic dinoflagellates is still poorly understood. It is hypothesized that dispersal might only occur via rafting. The molecular data suggest that the South China Sea *O. ovata* populations may have shared origin. Similarly, populations in the Malacca Straits may have a common origin with other populations that border the Indian Ocean. These populations may have evolved independently of each other over time, but there

Table 4. Pairwise Comparison of the 5.8S rRNA Gene, ITS1 and ITS2 Sequences of *Ostreopsis ovata* from Port Dickson, Kota Kinabalu, and Pulau Redang, Together with a Clone of *Ostreopsis lenticularis* from Pulau Redang*

			1	2	3	4	5	6	7	8	9	10	11	12	13
1	*O. ovata*	OvPD06		0.0000	0.0030	0.0870	0.0870	0.0870	0.0800	0.0899	0.0899	0.0899	0.0795	0.0730	0.5631
2		OvPD07	100		0.0030	0.0870	0.0870	0.0870	0.0800	0.0899	0.0899	0.0899	0.0795	0.0730	0.5631
3		OvPD04	99	99		0.0836	0.0836	0.0836	0.0766	0.0866	0.0866	0.0866	0.0762	0.0697	0.5631
4		OvPR04	89	89	89		0.0000	0.0000	0.0089	0.0149	0.0149	0.0149	0.0119	0.0180	0.5584
5		OvPR02	89	89	89	100		0.0000	0.0089	0.0149	0.0149	0.0149	0.0119	0.0180	0.5584
6		OvPR01	89	89	89	100	100		0.0089	0.0149	0.0149	0.0149	0.0119	0.0180	0.5584
7		OvPR03	92	92	92	99	99	99		0.0149	0.0149	0.0149	0.0059	0.0119	0.5457
8		OvSA02	90	90	91	98	98	98	98		0.0000	0.0000	0.0089	0.0210	0.5491
9		OvSA04	90	90	91	98	98	98	98	100		0.0000	0.0089	0.0210	0.5491
10		OvSA09	90	90	91	98	98	98	98	100	100		0.0089	0.0210	0.5491
11		OvSA10	92	92	92	98	98	98	99	99	99	99		0.0119	0.5428
12		OvSA06	92	92	93	98	98	98	98	97	97	97	98		0.5472
13	*O. lenticularis*	O1PR01	26	26	26	38	38	38	38	39	39	39	39	39	

*The upper right matrix indicates the Kimura 2-parameter distances between taxa. The lower left matrix shows the uncorrected percentage similarity between sequences.

Table 5. Pairwise Comparison Among Populations of *Ostreopsis ovata*

Population	Port Dickson	Kota Kinabalu	Pulau Redang
Port Dickson		0.0491	0.0496
Kota Kinabalu	0.9350		0.0111
Pulau Redang	0.9700	0.5420	

*Figures in the upper right panel are the index values of nucleotide diversity P_i (Nei, 1987), while those in the lower left panel are the values of F_{ST} (Hudson et al., 1992).

might have been insufficient time for genetic divergence to accumulate in these populations. Populations within a contiguous body of water may continue to intermix when seaweed debris from a particular location that have these dinoflagellates attached eventually settled on other seaweed beds. This may explain the genetic similarity between the Pulau Redang and Kota Kinabalu clones despite the large geographical separation. Clearly, many more isolates from different geographical areas need to be analyzed to get a more complete picture.

Sequence comparison of the ITS1 and ITS2 regions showed that the 3′ end of both regions (approx. 15 bp) are significantly different among the *O. ovata* isolates. Potential signature sequences can be found in the 3′ end of both ITS1 (positions 76–93) and ITS2 (positions 326–339). These variable regions may be useful as group-specific oligonucleotide probes.

The lengths of the 5.8S rRNA gene of *O. ovata* (159 bp) and *O. lenticularis* (160 bp) are closely similar to those of other dinoflagellates that have been studied. The ITS1 and ITS2 lengths, however, are significantly shorter than those of other dinoflagellates that have been reported (Adachi et al., 1996). Large differences in length and nucleotide sequences of ITS1 and ITS2 regions of *O. ovata* and *O. lenticularis* suggest that the regions are not suitable for delineation at the species level in *Ostreopsis,* although the regions have been used to differentiate between species of *Alexandrium* (Adachi et al., 1996). Variable ITS region sizes have also been observed in some other algal taxa (e.g., see Goff et al., 1994; Patwary et al., 1998). Some can be aligned at or above the generic level, and some can only be aligned poorly. Thus the differences in sequence alignability imply that the use of ITS regions as a taxonomic tool must be applied on a case-by-case basis (Connell, 2000).

The molecular data that we have generated in this study are the first of their kind for benthic/epiphytic dinoflagellates. Although the data are still limited, they do prove that a molecular approach can be highly useful in taxonomic and biogeographical studies of marine plankton. The data should also provide a useful comparison base for larger-scale biogeographical and taxonomic studies of these important dinoflagellates.

Acknowledgments

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